American Journal of Pathology, Vol. 152, No. 3, March 1998
Copyright © American Society for Investigative Pathology

Expression of Heme Oxygenase-1 in Atherosclerotic Lesions

Li-Ju Wang,* Tzong-Shyuan Lee,* Fan-Yen Lee,† Ru-Chun Pai,* and Lee-Young Chau*

From the Division of Cardiovascular Research, Institute of Biomedical Sciences, Academia Sinica, and Department of Cardiovascular Surgery†, Tri-Service General Hospital, Taipei, Taiwan, Republic of China*

Heme oxygenase-1 (HO-1) is a heme-degradation enzyme induced under various oxidative stress conditions. To elucidate the potential involvement of HO-1 in atherogenesis, the expression of this enzyme in atherosclerotic lesions of apolipoprotein E-deficient mice and humans were examined. Both immunostaining and *in situ* hybridization clearly demonstrated that the expression of HO-1 was prominent in endothelium and foam cells/macrophages of thickened intima in lesions from both humans and experimental animals. The expression of this enzyme was also detected in medial smooth muscle cells of advanced lesions. The induction of HO-1 mRNA was observed in murine peritoneal macrophages after treatment with oxidized low density lipoprotein (LDL) but not with native LDL in a dose-dependent manner. Time course study demonstrated that the induction was prominent at 3 hours, reached a maximal induction at 6 hours, and remained evident up to 24 hours after oxidized LDL treatment. The degree of induction was in concordant with the extent of oxidation in the LDL preparation. Lysophosphatidylcholine, one of the major components present in oxidized LDL, was ineffective to induce the gene expression, suggesting that other lipophilic substances derived from LDL oxidation are responsible for the induction of HO-1. These results clearly demonstrate that HO-1 is one of the stress proteins expressed in atherosclerotic lesions. *(Am J Pathol 1998, 152:711–720)*

Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism. Two HO isozymes derived from distinct genes were identified.[1] HO-1 is a stress-response protein and can be induced by a variety of oxidative-inducing agents including heme/hemoglobin, heavy metals, UV radiations, cytokines, and endotoxins.[2–6] In contrast, HO-2 is constitutively expressed and not inducible. The induction of HO-1 would lead to the degradation of pro-oxidant heme and production of antioxidant bilirubin, therefore it represents a cellular antioxidant defense mechanism.[7–9] Recently, increasing interest was drawn on the production of carbon monoxide, which is one of the products derived from HO reaction. It has been shown that CO shares some similar properties with nitric oxide. Both are capable of increasing cellular cGMP levels[10] and are suggested to be the retrograde messengers in the central nerve system.[11,12] Recently, the induction of HO-1, accompanied with the production of CO, was observed in vascular smooth muscle cells (VSMC) under hypoxia.[13] In addition to being a potential dilator of VSMC via cGMP-dependent mechanism,[13,14] the VSMC-derived CO was shown to suppress endothelin-1 and platelet-derived growth factor-B gene expression in endothelial cells and subsequently inhibit the proliferation of smooth muscle cells.[15] In view of the potential roles of HO-1 involving the pathophysiological conditions, in the present study we examined the expression of this enzyme in vascular lesions of atherosclerosis, which represents a chronic pathological process of multiple oxidative insults.[16,17] Our results clearly showed that HO-1 is expressed in the atherosclerotic vessels from humans and the apolipoprotein E (apoE)-deficient mice.[18,19] The macrophages appear to be the primary cell type in the thickened intima to express this enzyme. To additionally test whether the atherogenic substance, oxidized low density lipoprotein (LDL),[20] is able to induce the HO-1 gene expression in macrophages, *in vitro* study with murine peritoneal macrophages was performed. It was shown that HO-1 mRNA was rapidly up-regulated by treating cells with oxidized LDL but not native LDL, suggesting that the oxidized LDL is at least one of the stimuli *in vivo* to activate the HO-1 gene expression in atherosclerotic lesions.

Materials and Methods

Human Samples

Human ascending (n = 3) and abdominal (n = 5) aortas were obtained from patients (males, ages 53 to 80)

Supported by grants from the National Science Council of Taiwan (NSC 86–2314-B-001–003-M26) and Institute of Biomedical Sciences, Academia Sinica, Taiwan, Republic of China.

Accepted for publication December 10, 1997.

Address reprint requests to Lee-Young Chau, Division of Cardiovascular Research, Institute of Biomedical Sciences, Academia Sinica, Nankang, Taipei 11529, Taiwan, Republic of China. E-mail: lyc@ibms.sinica.edu.tw

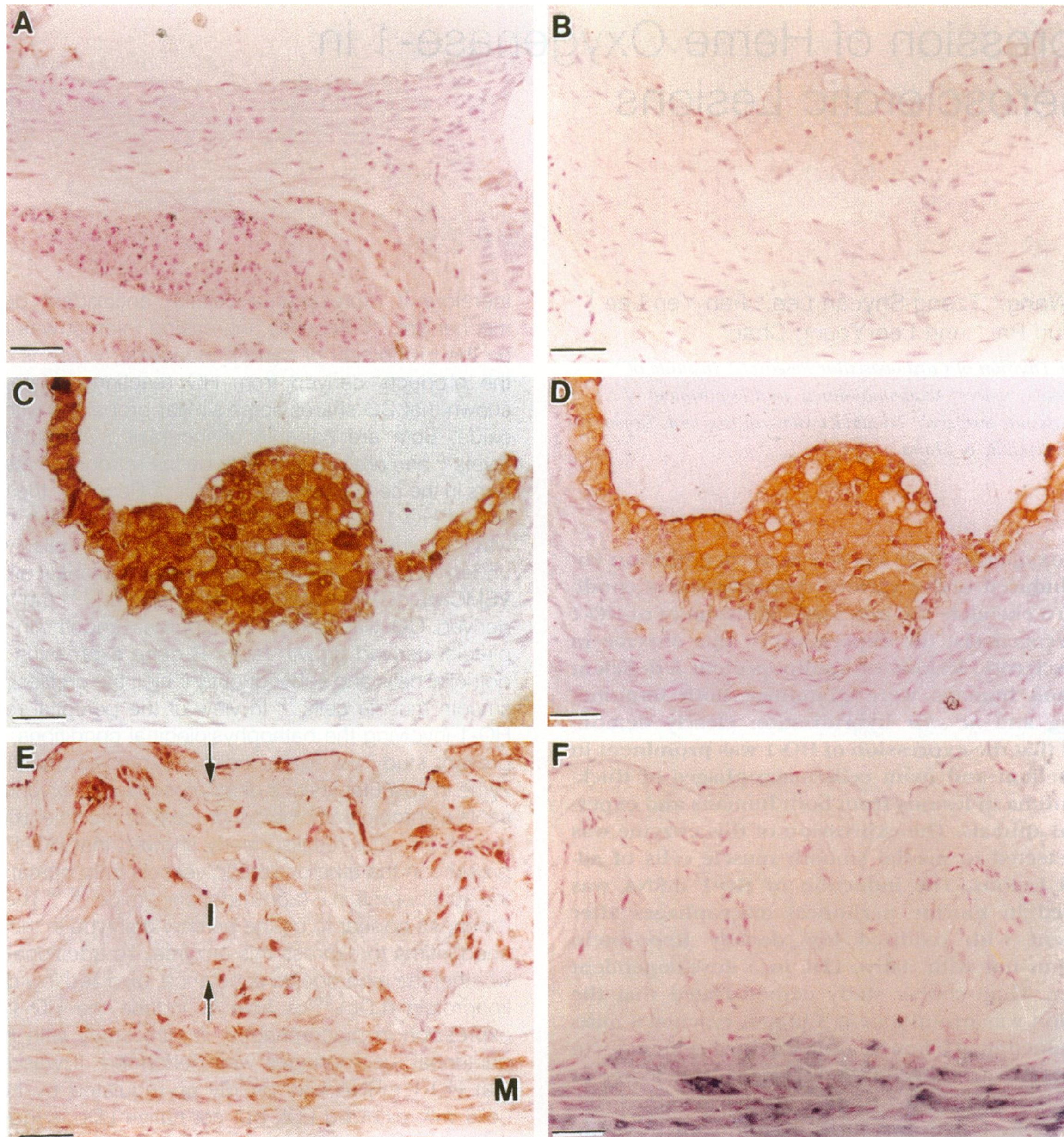

Figure 1. HO-1 immunostaining in atherosclerotic lesions of apoE-deficient mice. Normal aorta (**A**) and lesions of abdominal aortas from a 5-month-old-mouse (**C**) and a 6-month-old-mouse (**E**) were immunostained with rabbit antibody specific for HO-1 (dilution 1:200). Control section (**B**) incubated with normal rabbit IgG was negative. A consecutive section (**D**) from the same lesion of **C** was immunostained with antibody specific for mouse macrophages (F4/80, dilution 1:100). A consecutive section (**F**) from the same lesion of **E** was immunostained with antibody specific for smooth muscle cells (α-actin, dilution 1:50). All sections were counterstained with carmin red. The thickened intima (**I**) is marked by **arrows**. **M**, media. Magnification, ×200. Bars, 50 μm.

during surgery for ischemic heart disease or abdominal aortic aneurysmal disease. Immediately after surgery, tissue samples were rinsed with ice-cold phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde solution, and paraffin embedded. Tissues were serially sectioned at 5 μm and used for immunohistochemistry and *in situ* hybridization experiments thereafter. The sampling of patient specimens followed the procedure of the hospital's ethical committee, and the study was approved by the human subject review committee in our institute.

Animals

The homozygous apoE-deficient mice of C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, Maine) and maintained under conventional housing conditions in our animal facility. Mice (male, n = 6) fed with normal chow diet were killed between 4 to 6 months of age. Animals were anesthetized with an intraperitoneal injection of 0.1 ml of 2.5% sodium pentobarbital. After perfusion of aortic tree with ice-cold PBS, the heart and entire aorta were removed and immersed in 4%

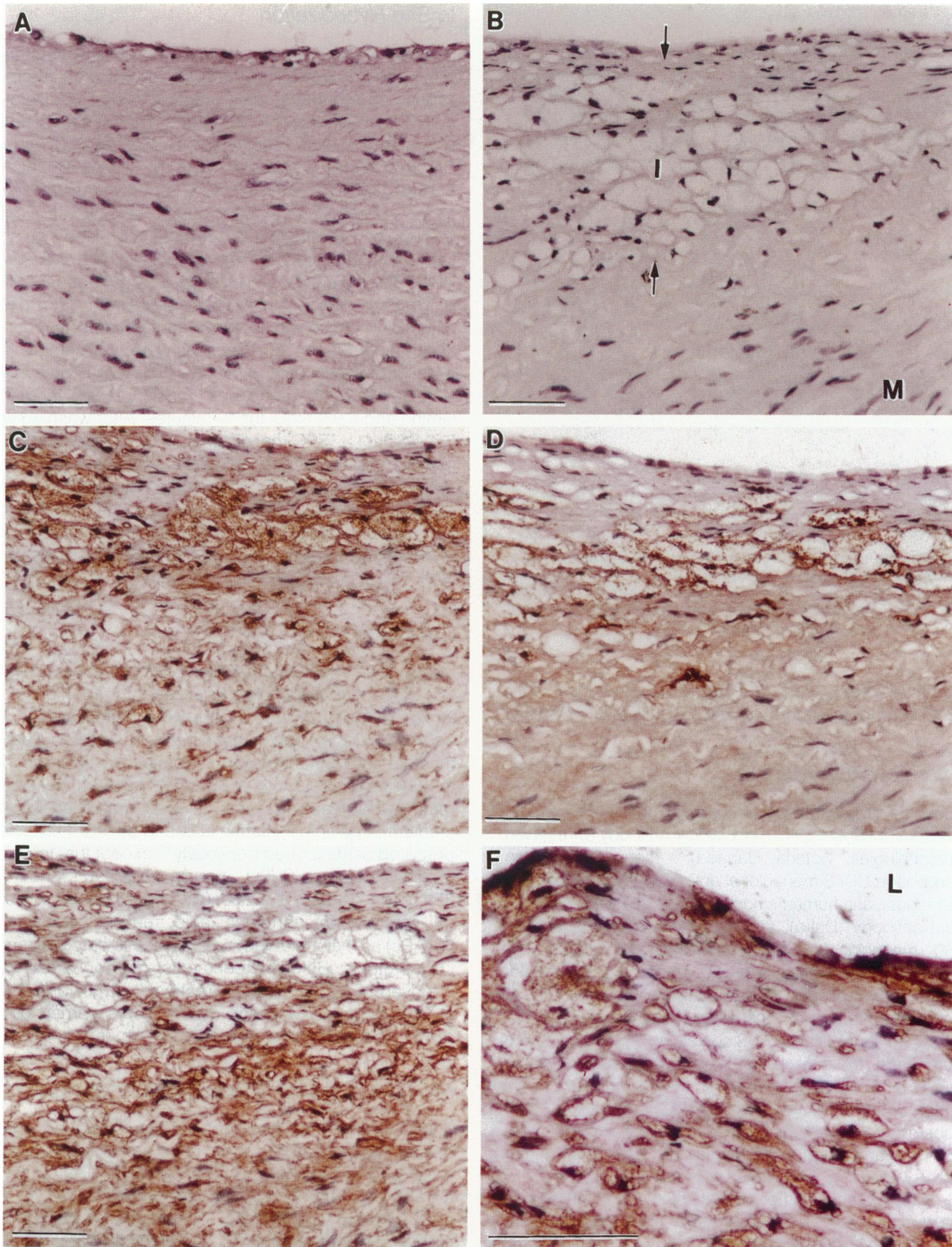

Figure 2. HO-1 immunostaining in human atherosclerotic lesion. The sections of human normal ascending aorta (**A**) and atherosclerotic abdominal aorta (**C** and **F**) were immunostained with antibody specific for HO-1 (dilution 1:200). The serial sections of abdominal aorta were immunostained with (**D**) antibody specific for human macrophages (CD68, dilution 1:200) and (**E**) antibody specific for smooth muscle cells (α-actin, dilution 1:100). Control section (**B**) incubated with normal rabbit IgG was negative. Sections were counterstained with hematoxylin. The intima (I) is marked by **arrows**. **M**, media; **L**, lumen. A-E, Magnification, ×200 (**A** to **E**); ×400 (**F**). Bars, 50 μm.

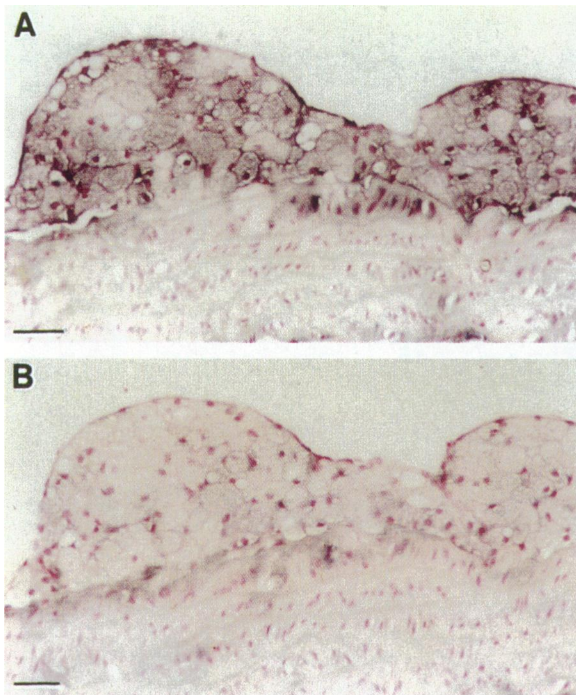

Figure 3. *In situ* hybridization of HO-1 mRNA in lesion of apoE-deficient mouse. Serial sections of atherosclerotic lesion from a 5-month-old mouse were hybridized with DIG-labeled HO-1 antisense RNA probe (**A**) or DIG-labeled HO-1 sense RNA probe (**B**), respectively. Sections were counterstained with carmin red. Magnification, ×200. Bars, 50 μm.

paraformaldehyde solution. The tissue sections were then prepared as described above for human samples.

Immunocytochemistry

Immunostaining was carried out using the following antibodies: rabbit anti-rat HO-1 polyclonal antibody (StressGen Biotechnologies, Victoria, Canada);[21] mouse anti-human macrophage CD68 monoclonal antibody (Dako, Kyoto, Japan); mouse anti-human smooth muscle cell α-actin monoclonal antibody (Dako); and rat anti-mouse macrophage F4/80 monoclonal antibody (Serotec, Kidlington, England).[22] Tissue sections were deparaffinized, rehydrated, and pretreated with 3% H_2O_2 in methanol for 10 minutes at room temperature to exhaust endogenous peroxidase activities. After incubation in PBS containing 1% bovine serum albumin and 1% goat serum at room temperature for 30 minutes, sections were treated with the first antibody for 30 minutes followed by 3 washes in PBS. Sections were then incubated with horseradish peroxidase-conjugated goat secondary antibody for 30 minutes at room temperature. After 3 washes in PBS, color was developed with 0.1% 3,3′-diaminobenzidine. For the detection of smooth muscle cells of mouse aortic sections, alkaline phosphatase-conjugated mouse anti-smooth muscle cell α-actin monoclonal antibody (Sigma, St. Louis, MO) was used, and the color was developed using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as substrate. Negative controls were performed by omission of the first antibody or substitution of normal rabbit IgG for HO-1 antibody or mouse IgG for monoclonal antibodies as the first antibodies.

In Situ *Hybridization*

Human HO-1 cDNA, which contains 333-bp sequence within the coding region, was prepared by reverse transcription-polymerase chain reaction using RNA isolated from human atherosclerotic aorta.[23] The primers used for polymerase chain reaction were 5′-CGACAGCATGC-CCCAGGATT-3′ (sense) and 5′- CGCTTCACAT-AGCGCTGCAT-3′ (antisense). The polymerase chain reaction product was subcloned into pCRII vector (Invitrogen, San Diego, CA), and the sequence was confirmed by DNA sequencing. The antisense and sense HO-1 RNAs were then synthesized by SP6 and T7 RNA polymerase, respectively, and labeled with digoxigenin (DIG)-UTP according to the manufacturer's instruction (Boehringer Mannheim). Sections were pretreated with 1 μg/ml proteinase K for 15 minutes at 37°C and acetylated with 0.25% acetic anhydride in 100 mmol/L triethanolamine and 0.9% NaCl for 10 minutes. After washing with 2× SSC (1× SSC consists of 150 mmol/L NaCl, 15 mmol/L sodium citrate, pH 7.0), sections were dehydrated with increasing concentration of ethanol and air-dried for 30 minutes. Hybridization was then carried out as described previously.[23] After completion of the color reaction, each section was counterstained with carmine red.

Cell Culture

Mouse peritoneal macrophages were harvested from C57BL/6 or apoE-deficient mice after intraperitoneal injection of thioglycolate.[23] Cells were plated at 2×10^6 cells per 60-mm dish with Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. After 2 hours of incubation at 37°C, nonadherent cells were removed by washing with Dulbecco's modified Eagle's medium three times. Human LDL and oxidized LDL were prepared as described previously[24] except that the incubation of LDL with $CuSO_4$ was prolonged to 24 hours at 37°C. The oxidized LDL contained approximately 30 to 60 nmol of thiobarbituric acid reactive substances (TBARs) as malondialdehyde equivalents per milligram of LDL proteins. Before experiments, macrophages were deprived of serum, and the treatment with oxidized LDL or lysophosphatidylcholine (lysoPC) was carried out in serum-free medium for the indicated times.

RNA Isolation and Northern Blot Analysis

Total RNA was isolated from cells by the acid guanidinium thiocyanate-phenol-chloroform extraction method described by Chomczynski and Sacchi.[25] The quality of the RNA was checked by the ratio of the A260/A280 and agarose gel electrophoresis. Equal amounts (5 to 10 μg) of total RNA were denatured and electrophoresed on 1% agarose gel containing 6.6% formaldehyde for 2 hours at 120 V. After electrophoresis, RNA was transferred onto nylon membrane and fixed by UV irradiation. Membranes were prehybridized at 42°C in solution containing 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), 50% formamide, 1 mol/L NaCl, and 1 mg/ml denatured soni-

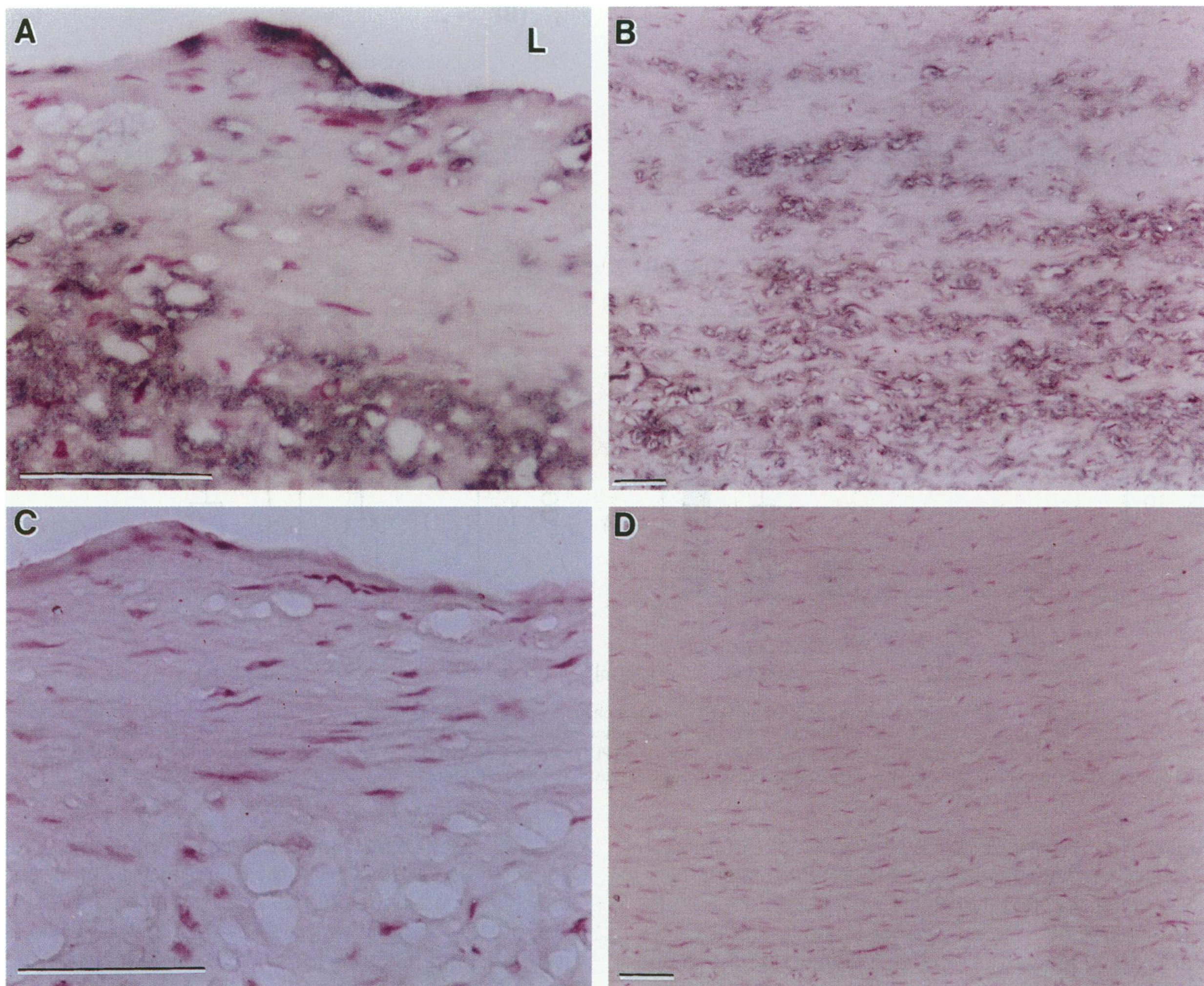

Figure 4. *In situ* hybridization of HO-1 mRNA in human atherosclerotic lesions. Tissue sections were hybridized with DIG-labeled HO-1 antisense RNA probe (**A** and **B**) or DIG-labeled HO-1 sense RNA probe (**C** and **D**), respectively, and counterstained with carmin red. Positive signals were detected in endothelium and macrophages of intima (**A**) and smooth muscle cells of the media (**B**). **L**, lumen. Magnification, ×400 (**A** and **C**); ×100 (**B** and **D**). Bars, 50 μm.

cated salmon sperm DNA for at least 1 hour. ^{32}P-labeled cDNA probes prepared by random priming at 1×10^6 cpm/ml were then added, and the hybridization continued overnight. Membranes were washed once with 2× SSC and 1% SDS for 30 minutes at 50°C and then washed twice with 0.2× SSC and 1% SDS at 50°C for 30 minutes each. The membranes were then exposed to Kodak XAR-5 film at −70°C using Kodak intensifying screen.

Western Blot

Cells (2×10^6) were rinsed with ice cold PBS twice and lysed in 62.5 mmol/L Tris-HCl, pH 6.8, containing 2% SDS, 2% 2-mercaptoethanol, and 5% glycerol and followed by boiling for 10 minutes. After sonication for 5 × 15 seconds using a microprobe sonicator (Ultrasonics W-375) at an output setting of 5 and a pulse cycle of 50%, 20 μg cell lysate proteins were electrophoresed on a 12% SDS-polyacrylamide gel and then transblotted onto Immobilon-P membranes (Millipore, Bedford, MA). The blots were blocked in PBS containing 0.1% Tween 20 and 5% skim milk at room temperature for 30 minutes. After two washes in PBS containing 0.1% Tween 20 (PBST buffer), blots were incubated with rabbit polyclonal anti-HO-1 antibody (dilution 1:1000) for another hour at room temperature in PBS containing 0.1% Tween 20 and 5% skim milk. After three washes in PBS containing 0.1% Tween 20, blots were incubated with alkaline phosphatase-conjugated goat anti-rabbit-IgG (Sigma, St. Louis, MO) (dilution 1:2500). After 1 hour at room temperature, blots were washed, and the color was developed using 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium as the substrate.

Results

Expression of Heme Oxygenase in Atherosclerotic Lesions

The expression of inducible HO-1 protein in atherosclerotic lesions of humans and apoE-deficient mice were examined using specific antibody for HO-1.[21] As shown

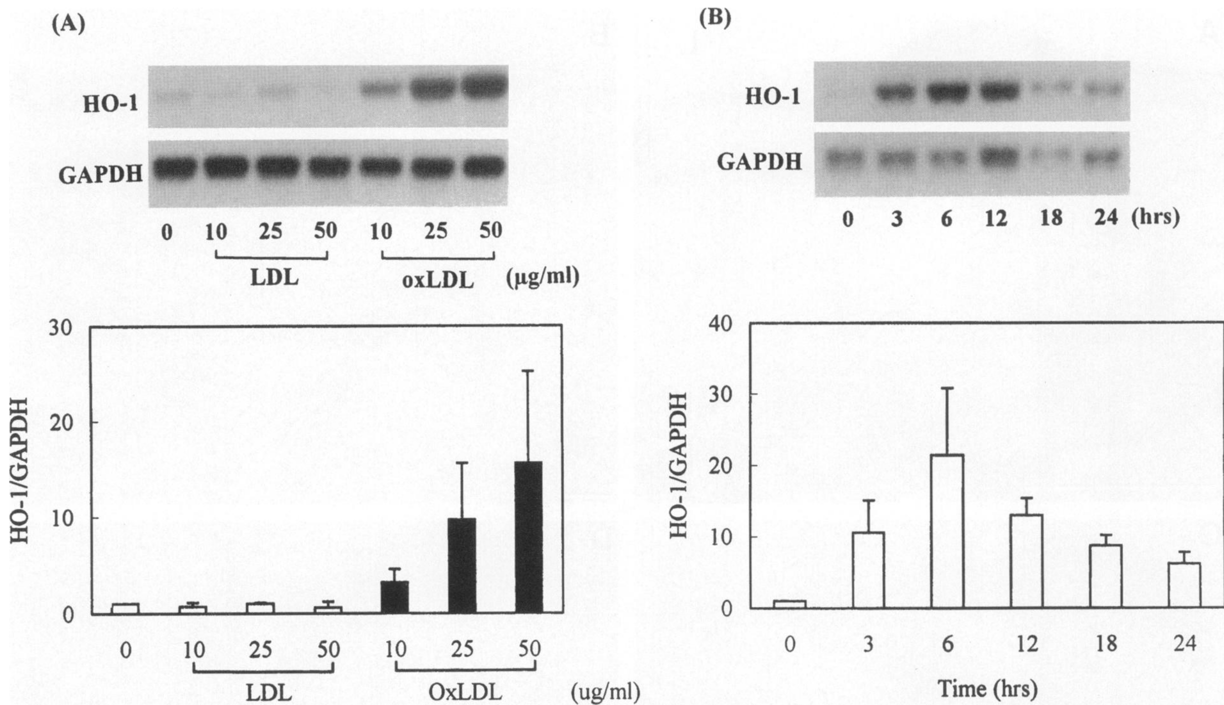

Figure 5. Induction of HO-1 mRNA in macrophages by oxidized LDL. **A:** Macrophages were treated with indicated concentrations of native LDL or oxidized LDL in culture for 6 hours. **B:** Cells were treated with oxidized LDL (50 μg/ml) for indicated times. The total RNA was isolated and Northern blot analysis with indicated cDNA probes was performed. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was served as an internal control. **Bar graph** shows quantitative data of three independent experiments obtained from densitometry analysis. The ratio of HO-1/GAPDH mRNA levels in control cells is referred to 1.

in Figure 1A, the immunoreactivity was not detected in sections of normal vessels from the apoE-deficient mice. In contrast, strong positive stains were detected in endothelium as well as in foam cells of the fatty streaks from the animals (Figure 1C). Immunostaining of the serial section with antibody specific for mouse macrophage F4/80 antigen[22] revealed that these foam cells were of the macrophage origin (Figure 1D). When the advanced lesions of experimental animals were examined, it was found that the HO-1 staining was also present in the smooth muscle cells of the media layer (Figure 1E). When experiments were performed with sections of human aortas, again no positive HO-1 stain was detected in sections of normal vessels (Figure 2A). Whereas, in sections with atherosclerotic lesions, the HO-1 immunostain (Figure 2C) was colocalized with that of the cell marker for macrophages in the intima area (Figure 2D). The positive stain was also detected in the medial smooth muscle cells as identified by immunostaining with specific antibody for SMC α-actin. (Figure 2E). When the immunostain was examined under higher magnification, the expression of HO-1 protein was also evident in endothelium as shown in Figure 2F. To additionally elucidate whether the increase of HO-1 protein is resulted from the induction of gene expression, *in situ* hybridization with antisense RNA to HO-1 gene was carried out. As demonstrated in Figure 3A, positive signals were strongly detected in the endothelium and macrophages of the fatty streak from experimental animals. Experiments conducted with the sense RNA were virtually negative (Figure 3B), indicating that the positive signals were specific. The expression of HO-1 mRNA in human lesions was shown in Figure 4. In parallel with the protein stain, the signals for HO-1 mRNA were evident in endothelial cells, foam cells/macrophages, and smooth muscle cells of the atherosclerotic aorta.

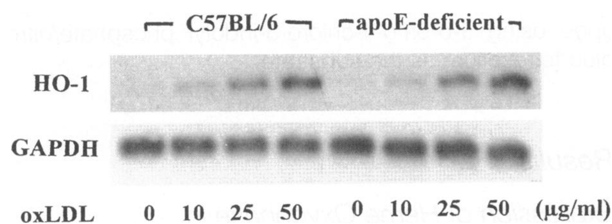

Figure 6. Induction of HO-1 mRNA by oxidized LDL in macrophages isolated from normal C57BL/6 and apoE-deficient mice. Peritoneal macrophages isolated from C57BL/6 or apoE-deficient mice were treated with indicated concentration of oxidized LDL for 6 hours. Total RNA was isolated and Northern blot analysis was performed. Result shown is the representative of three independent experiments.

Oxidized LDL-Induced Heme Oxygenase Gene Expression

To test whether the atherogenic substance, oxidized LDL, is capable of inducing the gene expression of HO-1, murine peritoneal macrophages isolated from C57BL/6 mice were treated with oxidized LDL and the effect on HO-1 mRNA level was assessed. As shown in Figure 5A, extensively oxidized LDL, but not native LDL, induced mRNA expression of HO-1 in these cells in a dose-dependent manner. Time course experiments additionally demonstrated that the increase in HO-1 mRNA occurred as early as 3 hours and reached a maximal around 6

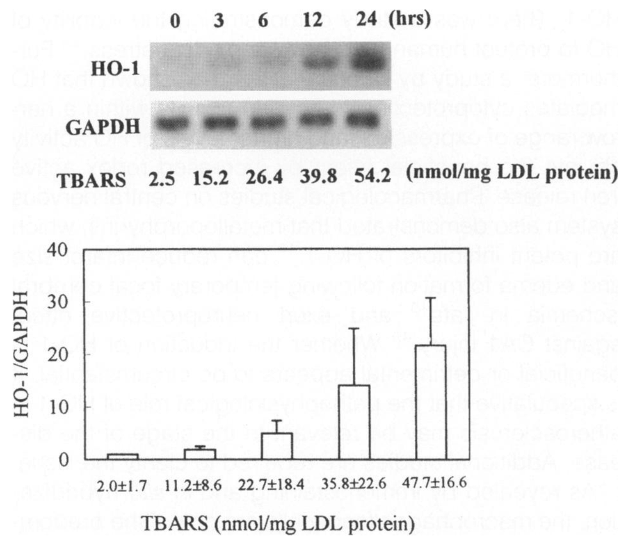

Figure 7. Effect of extent of oxidation in LDL on HO-1 mRNA induction in macrophages. LDL was incubated with 5 μmol/L $CuSO_4$ at 37°C for indicated times, and the TBAR value was determined. Cells were then treated with these oxidized LDLs (50 μg/ml/each) for 6 hours in culture. Total RNA was isolated, and the HO-1 mRNA level was analyzed by Northern blot. Bar graph shows quantitative data of four independent experiments.

hours after treatment (Figure 5B). Although it would then gradually decay, the induction remained evident up to 24 hours, which was the longest time point examined in the present study. The expression levels of HO-1 mRNA in peritoneal macrophages prepared from apoE-deficient mice were also examined. As shown in Figure 6, there was no significant difference in the basal levels of HO-1 mRNA in macrophages from C57BL/6 and apoE-deficient mice. Likewise, the degree of HO-1 induction following oxidized LDL treatment in macrophages of apoE-deficient mice was similar to that of C57BL/6 mice. This result indicates that the induction of HO-1 gene expression in apoE-deficient mice is not systemic. It has been shown that variable degrees of oxidation in LDL result in rather distinct biological activities.[26] We additionally examined the effect of the various extent of oxidation in LDL on the HO-1 induction. As shown in Figure 7, the degree of induction was proportional to the TBARs value of the oxidized LDL. The expression of HO-1 protein in murine macrophages following oxidized LDL treatment was additionally examined by Western blot. Consistent with the mRNA expression, the HO-1 protein was highly induced after 6 hours, and the level retained significantly higher than that of control cells after 24 hours following the oxidized LDL treatment in these cells (Figure 8). It has been shown that oxidation of LDL is associated with significant accumulation of lipid hydroperoxides, aldehydes, lysoPC, and a number of oxysterols.[27–29] As accumulative evidence has demonstrated that lysoPC is effective to regulate the expression of many genes and is responsible for parts of the biological activities of the oxidized LDL,[30–32] we examined the effect of lypoPC on the HO-1 gene expression in macrophages. It was noted that lysoPC at a high concentration (50 μg/ml) caused significant cell death following a 3-hour incubation (data not shown). Nevertheless, the HO-1 mRNA was not significantly induced in cells following 6 hours or 24 hours incubation with lysoPC at concentrations of up to 25 μg/ml, which did not affect the cell viability as assessed by trypan blue dye exclusion and detachment of the cells (data not shown). To explore the role of oxidative events in mediating the HO-1 induction by oxidized LDL, the effect of antioxidants on HO-1 gene expression was also assessed. As shown in Figure 9, pretreatment of cells with vitamin C, vitamin E, or iron chelator, desferrioxamine, before exposure to oxidized LDL did not significantly reduce the extent of HO-1 expression, suggesting that the oxidative mechanism(s) may not be responsible for the gene induction.

Discussion

In present study, we have demonstrated that HO-1 is highly induced in atherosclerotic lesions of humans and experimental animals. It is likely that the induction of HO-1 in atherosclerotic lesions may represent an adaptive response as observed in other pathophysiological conditions.[8,33,34] Several lines of evidence have supported the role of bilirubin, the end product derived from heme catabolism, as a physiological protectant against additional oxidative damage.[7,9,35–37] Furthermore, *in vitro* studies have shown that bilirubin is an efficient antioxidant to inhibit oxidation of low density lipoprotein,[38,39] which is one of the important events in atherogenesis.[16,17] Recently, *in vitro* studies on cultured vascular endothelial cells have shown that overexpression of HO by pre-exposure to oxidants or transfection of HO gene could protect cells from subsequent oxidative insults.[40,41] It is envisaged that the induction of HO-1 and the production of bilirubin in early lesions may have a beneficial role in reducing the oxidative reactions and attenuating the subsequent progression of the plaques. In addition to bilirubin, the CO produced during the degradation of heme has recently attracted a great deal of interest on its potential function in regulating the vascular tone.[13,14] It is apparent that the physiological roles of these heme degradation products implicated in the atherogenesis are worth additional investigation.

Evidence is accumulating that the synthesis of apoferritin protein is up-regulated posttranscriptionally following the induction of HO-1, presumably mediated by the iron released from heme degradation in cells subjected to oxidative insults.[40,42–44] The apoferritin, which can function as an intracellular sequestrator for noxious free iron, accounts for part of the antioxidative response following the HO-1 induction.[40,42–44] A recent study from our laboratory has shown that apoferritin protein is highly induced in early atherosclerotic lesions.[24] Although the induction appears to be regulated at a transcriptional level, the possibility that some of the ferritin protein synthesized is secondary to the HO-1 induction cannot be ruled out. It is believed that HO-1 and apoferritin induced in early atherosclerotic lesions may function cooperatively to protect cells from additional oxidative damage by iron overload or other stress conditions. Recently however, a few reports did not support the protective role of

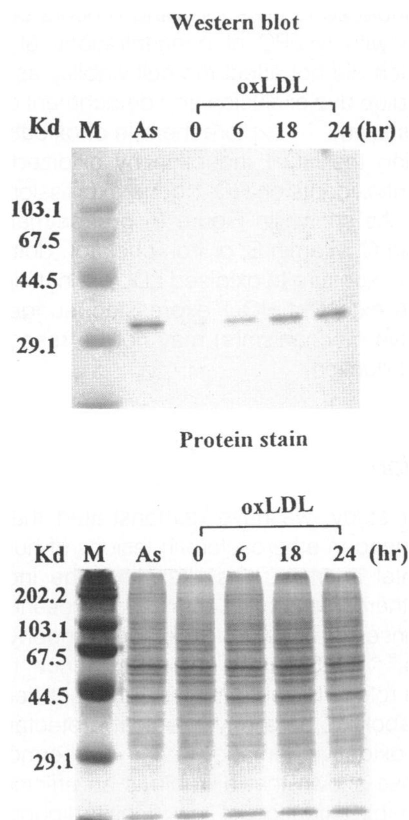

Figure 8. Western blot analysis of HO-1 protein expression in murine macrophages following oxidized LDL treatment. Cells were treated without (control) or with oxidized LDL (50 μg/ml) for indicated times. Cell lysates were prepared and subjected to SDS-polyacrylamide gel electrophoresis. Immunoblotting was performed with antibody specific for HO-1 (dilution, 1:1000). Cells treated with 50 μmol/L sodium arsenite (**As**) for 6 hours were included as a positive control. The equality of protein loading in each lane was demonstrated by Coomassie blue stain. **M**, prestained protein markers. Data shown are representatives of three independent experiments.

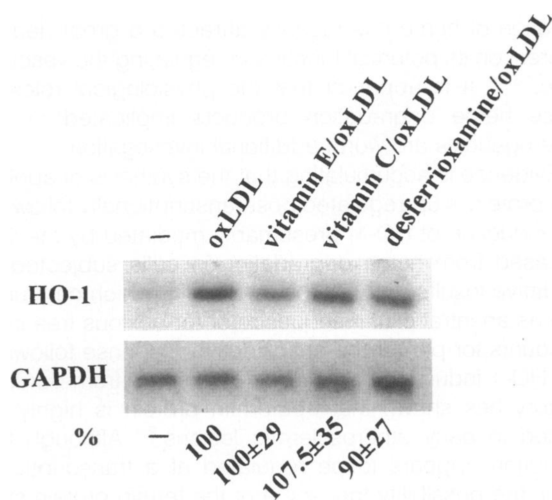

Figure 9. Effect of antioxidants on HO-1 mRNA expression in macrophages. Cells were pretreated without (control) or with vitamin E (100 μmol/L), vitamin C (200 μmol/L), and desferrioxamine (500 μmol/L) in culture for 1 hour. Cells were then exposed to oxidized LDL (25 μg/ml) with or without antioxidants for another 6 hours. Total RNA was isolated, and Northern blot analysis with indicated cDNA probes was performed. Blot shown is a representative result. The HO-1 induction in control cells was designated as 100%. Data presented are the mean ± SD of four independent experiments.

HO-1. There was a study demonstrating the inability of HO to protect human cells against oxidant stress.[45] Furthermore, a study by Dennery et al[46] has shown that HO mediates cytoprotection to oxygen toxicity within a narrow range of expression, and higher levels of HO activity obviate the beneficial effect by increased redox active iron release. Pharmacological studies on central nervous system also demonstrated that metalloporphyrins, which are potent inhibitors of HO-1,[47] can reduce infarct size and edema formation following temporary focal cerebral ischemia in rats[48] and exert neuroprotective effect against CA1 injury.[49] Whether the induction of HO-1 is beneficial or detrimental appears to be circumstantial. It is speculative that the pathophysiological role of HO-1 in atherosclerosis may be relevant to the stage of the disease. Additional studies are required to clarify the issue.

As revealed by immunostaining and *in situ* hybridization, the macrophages/foam cells are one of the predominant cell types to express HO-1 in atherosclerotic plaques. To identify the oxidative agent(s) responsible for the induction of HO-1 in atherosclerotic lesions, the murine macrophages were used as a cell model to examine the effect of the atherogenic substance, oxidized LDL, on the HO-1 gene expression. It was of great interest to find that oxidized LDL, but not native LDL, is a strong inducer of this enzyme. The induction was prominent within 3 hours after stimulation, implicating that activation of the gene transcription may be the underlying mechanism responsible for the induction, as observed in the cases of other oxidative agents.[50–52] Although the extent of oxidative modifications of LDL, as revealed by the TBAR values, appeared to be associated with the degree of HO-1 induction, pretreatment of cells with antioxidants and iron chelator did not attenuate the HO-1 gene expression induced by oxidized LDL. This result suggests that the gene induction is likely mediated by the product(s) derived from the oxidation of LDL but not the oxidative events per se. Very recently, a study by Ishikawa et al[53] demonstrated that products of oxidized arachidonic acid-containing phospholipid induces HO-1 gene expression in co-cultures of human endothelial cells and smooth muscle cells. As lysoPC, one of the major components present in the oxidized LDL, was inactive to induce the HO-1 gene expression, it is possible that other components such as lipid hydroperoxides, lipid aldehydes, or oxysterols[27–29] may be the potential candidate substances responsible for the activity of oxidized LDL. An earlier report by Siow et al[54] also demonstrated the induction of HO-1 by oxidized LDL in VSMCs. Recently, it has been shown that the HO-1 is highly induced in smooth muscle cells under hypoxia. As the expression of HO-1 in smooth muscle cells is more evident in advanced lesions, it will be of great interest to know whether hypoxia is the main cause leading to the induction in smooth muscle cells in the late-stage of the disease.[13] Furthermore, because the endothelium represents the vascular lining proximate to the circulating red blood cells, it is conceivable that heme/hemoglobin released from damaged red blood cells may elicit the induction of HO-1 in endothelial cells, as proposed by other investigators.[33,40] However, a recent study by Agarwal et al[55] also demon-

strated that oxidized LDL is one of the agents to mediate the induction of HO-1 in endothelial cells. Together with this study, it clearly revealed that oxidized LDL is one of the potent agents responsible for the induction of HO-1 in atherosclerotic lesions.

In summary, the present study has demonstrated that HO-1 is one of the stress proteins induced in atherosclerotic lesions. Oxidized LDL is a potent inducer for the HO-1 gene expression in endothelial cells, macrophages, and smooth muscle cells. Although accumulative evidence has favored the protective role of HO against oxidative injury, the pathological relevance of the HO-1 induction in the development of the chronic vascular disease remains to be clarified.

References

1. Cruse I, Maines MD: Evidence suggesting that the two forms of heme oxygenase are products of different genes. J Biol Chem 1988, 263: 3348–3353
2. Yoshida T, Biro P, Cohen T, Muller RM, Shibahara S: Human heme oxygenase cDNA and induction of its mRNA by heme. Eur J Biochem 1988, 171:457–461
3. Alam J, Shibahara S, Smith A: Transcriptional activation of the heme oxygenase gene by heme and cadmium in mouse heptoma cells. J Biol Chem 1989, 264:6371–6375
4. Keyse SM, Tyrrell RM: Induction of the heme oxygenase gene in human skin fibroblasts by hydrogen peroxide and UVA (365 nm) radiation:evidence for the involvement of the hydroxyl radical. Carcinogenesis 1990, 11:787–791
5. Rizzardini M, Terao M, Falciani F, Cantoni L: Cytokine induction of haem oxygenase mRNA in mouse liver. Biochem J 1993, 290:343–347
6. Cantoni L, Rossi C, Riardini M, Gadina M, Ghezzi P: Interleukin-1 and tumor necrosis factor induce hepatic haem oxygenase: feedback regulation by glucocorticoids. Biochem J 1991, 279:891–894
7. Stocker R, Yamamoto Y, McDonagh AF, Glazer AN, Ames BN: Bilirubin is an antioxidant of possible physiological importance. Science 1987, 235:1043–1046
8. Llesuy SF, Tomaro ML: Heme oxygenase and oxidative stress: evidence of involvement of bilirubin as physiological protector against oxidative damage. Biochim Biophys Acta 1994, 1223:9–14
9. Stocker R: Induction of heam oxygenase as a defense against oxidative stress. Free Radical Res Commun 1990, 9:101–112
10. Schmidt HH: NO, CO,and HO endogenous soluble guanylyl cyclase-activating factors. FEBS Lett 1992, 307:102–107
11. Hawkins RD, Zhuo M, Arancio O: Nitric oxide and carbon monoxide as possible retrograde messengers in hippocampal long-term potentiation. J Neurobiol 1994, 25:652–665
12. Verma A, Hirsch DJ, Glatt CE, Ronnett GV, Snyder SH: Carbon monoxide: a putative neural messenger. Science 1993, 259:381–384
13. Morita T, Perrella MA, Lee M-E, Kourembanas S: Smooth muscle cell-derived carbon monoxide is a regulator of vascular cGMP. Proc Natl Acad Sci USA 1995, 92:1475–1479
14. Furchgott RF, Jothianandan D: Endothelium-dependent and independent vasodilation involving cyclic GMP: relaxation induced by nitric oxide, carbon monoxide and light. Blood Vessels 1991, 28:52–61
15. Morita T, Kourembanas S: Endothelial cell expression of vasoconstrictors and growth factors is regulated by smooth muscle cell-derived carbon monoxide. J Clin Invest 1995, 96:2676–2682
16. Ross R: The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993, 362:801–809
17. Berliner JA, Navab M, Fogelman AM, Frank JS, Demer LL, Edward PA, Watson AD, Lusis AJ: Atherosclerosis: basic mechanisms, oxidation, inflammation, and genetics. Circulation 1995, 91:2488–2496
18. Plump AS, Smith JD, Hayek T, Aalto-Setala K, Walsh A, Verstuyft JG, Rubin EM, Breslow JL: Severe hypercholesterolemia and atherosclerosis in apolipoprotein E -deficient mice created by homologous recombination in ES cells. Cell 1992, 71:343–353
19. Zhang SH, Reddick RL, Piedrahita JA, Maeda N: Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. Science 1992, 258:468–471
20. Witztum JL: The oxidation hypothesis of atherosclerosis. Lancet 1994, 344:793–795
21. Smith MA, Kutty RK, Richey PL, Yan S-D, Stern D, Chader GJ, Wiggert B, Petersen RB, Perry G: Heme oxygenase-1 is associated with the neurofibrillary pathology of Alzheimer's disease. Am J Pathol 1994, 145:42–47
22. Qiao J-H, Xie P-Z, Fishbein MC, Kreuzer J, Drake TA, Demer LL, Lusis AJ: Pathology of atheromatous lesions in inbred and genetically engineered mice: genetic determination of arterial calcification. Arterioscler Thromb 1994, 14:1480–1497
23. Renier G, Skamene E, DeSanctis JB, Radzioch D: High macrophage lipoprotein lipase expression and secretion and associated in inbred murine strains with susceptibility to atherosclerosis. Arterioscler Thromb 1993, 13:190–196
24. Pang J-HS, Jiang M-J, Chen Y-L, Wang F-W, Wang DL, Chu S-H, Chau L-Y: Increased ferritin gene expression in atherosclerotic lesions. J Clin Invest 1996, 97:2204–2212
25. Chomczynski P, Sacchi N: Single-step method of RNA isolated by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987, 162:156–159
26. Björkerud B, Björkerud S: Contrary effects of lightly and strongly oxidized LDL with potent promotion of growth versus apoptosis on arterial smooth muscle cells, macrophages, and fibroblasts. Arterioscler Thromb Vasc Biol 1996, 16:416–424
27. Lenz ML, Hughes H, Mitchell JR, Via DP, Guyton JR, Taylor AA, Gotto AM Jr, Smith CV: Lipid hydroperoxy and hydroxy derivatives in copper-catalyzed oxidation of low density lipoprotein. J Lipid Res 1990, 31:1043–1050
28. Esterbauer H, Jurgens G, Quehenberger O, Koller E: Autoxidation of human low density lipoprotein: loss of polyunsaturated fatty acids and vitamin E and generation of aldehydes. J Lipid Res 1987, 28:495–509
29. Jialal I, Freeman DA, Grundy SM: Varying susceptibility of different low density lipoproteins to oxidative modification. Arterioscler Thromb 1991, 31:1361–1369
30. Sakai M, Miyazaki A, Hakamata H, Sasaki T, Yui S, Yamazaki M, Schichiri M, Horiuchi S: Lysophosphatidylcholine plays an essential role in the mitogenic effect of oxidized low density lipoprotein on murine macrophages. J Biol Chem 1994, 269:31430–31435
31. Nakano T, Raines EW, Abraham JA, Klagsbrun M, Ross R: Lysophosphatidylcholine upregulates the level of heparin-binding epidermal growth factor-like growth factor mRNA in human monocytes. Proc Natl Acad Sci USA 1994, 91:1069–1074
32. Chai Y-C, Howe PH, DiCorleto PE, Chisolm GM: Oxidized low density lipoprotein and lysophosphatidylcholine stimulate cell cycle entry in vascular smooth muscle cells: evidence for release of fibroblast growth factor-2. J Biol Chem 1996, 271:17791–17797
33. Nath KA, Balla G, Vercellotti GM, Balla J, Jacob HS, Levitt MD, Rosenberg ME: Induction of heme oxygenase is a rapid, protective response in rhabdomyolysis in the rat. J Clin Invest 1992, 90:267–270
34. Balla J, Nath KA, Balla G, Juckett MB, Jacob HS, Vercellotti GM: Endothelial cell heme oxygenase and ferritin induction in rat lung by hemoglobin in vivo. Am J Physiol 1995, 268:L321–L327
35. Neuzil J, Stocker R: Bilirubin attenuates radical-mediated damage to serum albumin. FEBS Lett 1993, 331:281–284
36. Yamaguchi T, Horio F, Hashizume T, Tanaka M, Ikeda S, Kakinuma A, Nakajima H: Bilirubin is oxidized in rats treated with endotoxin and acts as a physiological antioxidant synergistically with ascorbic acid in vivo. Biochem Biophys Res Commun 1995, 214:11–19
37. Yamaguchi T, Terakada M, Horio F, Aoki K, Tanaka M, Nakajima H: Role of bilirubin as an antioxidant in an ischemia-reperfusion of rat liver and induction of heme oxygenase. Biochem Biophys Res Commun 1996, 223:129–135
38. Neuzil J, Stocker R: Free and albumin-bound bilirubin are efficient co-antioxidants for α-tocopherol, inhibiting plasma and low density lipoprotein lipid peroxidation. J Biol Chem 1994, 269:16712–16719
39. Wu TW, Fung KP, Wu J, Yang CC, Weisel RD: Antioxidation of human low density lipoprotein by unconjugated and conjugated bilirubins. Biochem Pharmacol 1996, 51:859–862
40. Balla J, Jacob HS, Balla G, Nath K, Eaton JW, Vercellotti GM: Endothelial cell heme uptake from heme proteins: induction of sensitization

and desensitization to oxidant damage. Proc Natl Acad Sci USA 1993, 90:9285–9289

41. Abraham NG, Lavrovsky Y, Schwartzman ML, Stoltz RA, Levere RD, Gerritsen ME, Shibahara S, Kappas A: Transfection of the human oxygenase gene into rabbit coronary microvessel endothelial cells: protective effect against heme and hemoglobin toxicity. Proc Natl Acad Sci USA 1995, 92:6798–6802
42. Vile GF, Tyrrell RM: Oxidative stress resulting from ultraviolet A irradiation of human skin fibroblasts leads to a heme oxygenase-dependent increase in ferritin. J Biol Chem 1993, 268:14678–14681
43. Balla G, Jacob HS, Balla J, Rosenberg M, Nath K, Apple F, Eaton JW, Vercellotti GM: Ferritin: a cytoprotective antioxidant strategem of endothelium. J Biol Chem 1992, 267:18148–18153
44. Vile GF, Basu-Modak S, Waltner C, Tyrrell RM: Heme oxygenase 1 mediates an adaptive response to oxidative stress in human skin fibroblasts. Proc Natl Acad Sci USA 1994, 91:2607–2610
45. Nutter LM, Sierra EE, Ngo EO: Heme oxygenase does not protect human cells against oxidant stress. J Lab Clin Med 1994, 123:506–514
46. Dennery PA, Sridhar KJ, Lee CS, Wong HE, Shokoohi V, Rodgers PA, Spitz DR: Heme oxygenase-mediated resistance to oxygen toxicity in hamster fibroblasts. J Biol Chem 1997, 272:14937–14942
47. Maines MD: Zinc-protoporphyrin is a selective inhibitor of heme oxygenase activity in the neonatal rat. Biochim Biophys Acta 1981, 673:339–350
48. Kadoya C, Domino EF, Yang G-Y, Stern JD, Betz AL: Preischemic but not postischemic zinc protoporphyrin treatment reduces infarct size and edema accumulation after temporary focal cerebral ischemia in rats. Stroke 1995, 26:1035–1038
49. Panizzon KL, Dwyer BE, Nishimura RN, Wallis RA: Neuroprotection against CA1 injury with metalloporphyrins. NeuroReport 1996, 7:662–666
50. Keyse SM, Applegate LA, Tromvoukis Y, Tyrrell RM: Oxidant stress leads to transcriptional activation of the human heme oxygenase gene in cultured skin fibroblasts. Mol Cell Biol 1990, 10:4967–4969
51. Alam J: Multiple elements within the 5′-distal enhancer of the mouse heme oxygenase -1 gene mediate induction by heavy metals. J Biol Chem 1994, 269:2049–25056
52. Lavrovsky Y, Schwartzman ML, Levere RD, Kappas A, Abraham NG: Identification of binding sites for transcriptional factors NF-kappa B and AP-2 in the promoter region of the human heme oxygenase 1 gene. Proc Natl Acad Sci USA 1994, 91:5987–5991
53. Ishikawa K, Navab M, Leitinger N, Fogelman AM, Lusis AJ: Induction of heme oxygenase-1 inhibits the monocyte transmigration induced by mildly oxidized LDL. J Clin Invest 1997, 100:1209–1216
54. Siow RCM, Ishii T, Sato H, Taketani S, Leake DS, Sweiry JH, Pearson JD, Bannai S, Mann GE: Induction of the antioxidant stress proteins heme oxygenase-1 and MSP23 by stress agents and oxidised LDL in cultured vascular smooth muscle cells. FEBS Lett 1995, 368:239–242
55. Agarwal A, Balla J, Balla G, Croatt AJ, Vercellotti GM, Nath KA: Renal tubular epithelial cells mimic endothelial cells upon exposure to oxidized LDL. Am J Physiol 1996, 271:F814–F823